Freezing Sensitivity in the *sfr4* Mutant of Arabidopsis Is Due to Low Sugar Content and Is Manifested by Loss of Osmotic Responsiveness[1]

Matsuo Uemura*, Gareth Warren, and Peter L. Steponkus[2]

Department of Crop and Soil Sciences, Cornell University, Ithaca, New York 14853 (M.U., P.L.S.); Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Morioka 020–8550, Japan (M.U.); and School of Biological Sciences, Royal Holloway, University of London, Egham TW20 0EX, United Kingdom (G.W.)

Protoplasts were tested to determine whether the freezing sensitivity of the *sfr4* (sensitive to freezing) mutant of Arabidopsis was due to the mutant's deficiency in soluble sugars after cold acclimation. When grown under nonacclimated conditions, *sfr4* protoplasts possessed freezing tolerance similar to that of wild type, with the temperature at which 50% of protoplasts are injured (LT_{50}) of −4.5°C. In both wild-type and *sfr4* protoplasts, expansion-induced lysis was the predominant lesion between −2°C and −4°C, but its incidence was low (approximately 10%); below −5°C, loss of osmotic responsiveness (LOR) was the predominant lesion. After cold acclimation, the LT_{50} was decreased to only −5.6°C for *sfr4* protoplasts, compared with −9.1°C for wild-type protoplasts. Although expansion-induced lysis was precluded in both types of protoplasts, the *sfr4* protoplasts remained susceptible to LOR. After incubation of seedlings in Suc solution in the dark at 2°C, freezing tolerance and the incidence of freeze-induced lesions in *sfr4* protoplasts were examined. The freezing tolerance of isolated protoplasts (LT_{50} of −9°C) and the incidence of LOR were now similar for wild type and *sfr4*. These results indicate that the freezing sensitivity of cold-acclimated *sfr4* is due to its continued susceptibility to LOR (associated with lyotropic formation of the hexagonal II phase) and associated with the low sugar content of its cells.

Many plants increase their tolerance to freezing when exposed to low but nonfreezing temperatures in an adaptive process known as cold acclimation. During cold acclimation, numerous biochemical and physiological changes occur. Notable changes include alterations in membrane composition (Steponkus et al., 1993); accumulation of compatible solutes such as soluble sugars, Pro, and betaine (Guy, 1990); and altered gene expression (for review, see Thomashow, 1999). Although seemingly disparate, many of these changes are thought to contribute to the increase in the cryostability of cellular membranes. Increased membrane cryostability is a prerequisite for freezing tolerance because freeze-induced destabilization of cellular membranes is the primary cause of injury in plants (Steponkus, 1984; Steponkus et al., 1993). We have been able to distinguish several types of freeze-induced membrane lesions, including expansion-induced lysis (EIL) and loss of osmotic responsiveness (LOR; Steponkus et al., 1988; Uemura and Steponkus, 1989; Uemura et al., 1995).

Accumulation of soluble sugars during cold acclimation has been demonstrated in many plant species, including both herbaceous and woody plants (for review, see Levitt, 1980; Sakai and Larcher, 1987). Under natural conditions, soluble sugars increase during the onset of winter, when plants are subjected to low temperatures; conversely, soluble sugars decrease in spring when plants are deacclimating (Sakai, 1962; Siminovitch, 1981). Sugar content also increases during cold acclimation under artificial conditions (Koster and Lynch, 1992; Sasaki et al., 1996). Recently, it has been also shown that this is true for Arabidopsis, the experimental material in the present study (Ristic and Ashworth, 1993; McKown et al., 1996; Wanner and Junttila, 1999).

Despite the results documented above, the role of the accumulated sugars in freezing tolerance in planta remains unresolved. In one study, wheat (*Triticum aestivum*) varieties that differed in their freezing tolerance failed to show a positive correlation between sugar content and freezing tolerance; among a small group of cultivars, there even appeared to be a negative correlation (Green and Ratzlaff, 1975). On the other hand, another study reported a relatively good positive correlation between freezing tolerance and soluble sugar contents in 18

[1] This work was supported by the U.S. Department of Agriculture/National Research Initiative Competitive Grants Program (grant no. 96–35100–3163 to P.L.S.), by the U.S. Department of Energy (grant no. DE–FG01–84ER13214 to P.L.S.), by the UK Biotechnology and Biological Sciences Research Council (grant to G.W.), and by the Bio-oriented Technology Research Advancement Institution, Japan (grant to M.U.).

[2] Professor Steponkus died before the final drafting of this manuscript.

* Corresponding author; e-mail uemura@iwate-u.ac.jp; fax 81–19–621–6253.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.102.013227.

wheat cultivars (Yoshida et al., 1998). Such discrepancies might have arisen because some studies have correlated the changes in sugar content and freezing tolerance during cold acclimation, whereas others have compared the fully acclimated levels of both. It is desirable to determine the direct effect of sugars on the incidence of freeze-induced lesions, sidestepping some of the shortcomings of the correlative approach.

We have isolated several mutant lines of Arabidopsis that are impaired in their freezing tolerance after cold acclimation, the *sfr* (sensitivity to freezing) mutants. Their freezing tolerance before cold acclimation is unaffected (Warren et al., 1996), in contrast to mutants such as *aba* (Heino et al., 1990; Gilmour and Thomashow, 1991; Mäntylä et al., 1995) and *hos2* (Lee et al., 1999). This allows the freezing sensitivity of the *sfr* mutants to be specifically attributed to defects in the acclimation process, rather than generalized debilitation.

Quantitative examination of freezing tolerance by the electrolyte leakage assay showed that all the *sfr* mutants actually gained in freezing tolerance during cold acclimation, but to a lesser extent than the wild type. The deficit was greatest in the *sfr4* mutant, in accord with its high degree of sensitivity in survival tests on intact plants (Warren et al., 1996). Subsequently, it was observed that *sfr4*, alone among the mutants, underwent a reduction in its Suc and Glc contents, in contrast to the increase shown by wild type during cold acclimation (McKown et al., 1996). This suggested that the lack of sugar accumulation might be sufficient to account for the impaired freezing tolerance of *sfr4* after cold acclimation. Thus, the *sfr4* mutation may be useful for studying the role of sugars in protection against freezing injury. However, a causal relationship between the reduced sugar content and the impaired freezing tolerance in *sfr4* mutant still needed to be established.

We have developed an experimental system to artificially manipulate the sugar content by incubation of seedlings of Arabidopsis in Suc solution (Uemura and Steponkus, 1997, 2003), which is a modification of the method of Tumanov and Trunova (1963). After dark incubation of plants in a 400 mM Suc solution for 7 d at 2°C, the freezing tolerance of isolated protoplasts increases to a level similar to that elicited by normal (illuminated) cold acclimation. Moreover, the incidences of particular freeze-induced lesions such as EIL and LOR are similar to those after natural cold acclimation. We conclude that this protocol is useful to manipulate cellular sugar content, and, thus, to investigate the effect of sugar content on the incidence of freeze-induced lesions. Using this approach, the present study has aimed: (a) to explain the freezing sensitivity of *sfr4* in terms of particular types of freeze-induced lesions, and (b) to discover whether the reduced sugar contents in *sfr4* is the cause of its freezing sensitivity.

RESULTS

Effect of Cold Acclimation on Freezing Tolerance of Protoplasts

When the source plants were grown under nonacclimating conditions (continuous illumination at 23°C), protoplasts isolated from the *sfr4* mutant showed a freezing tolerance similar to that of protoplasts isolated from wild type (Fig. 1). In both types of protoplasts, survival declined from >90% to <10% over the range of −2°C to −6°C, with the temperature at which 50% of protoplasts are injured (LT_{50}) of approximately −4.5°C. After cold acclimation of the source plants for 7 d (2°C, 8-h illumination), protoplasts of both types displayed an increase in freezing tolerance, but the increase was significantly greater for wild type than for *sfr4*. In wild-type protoplasts isolated from cold-acclimated (ACC) leaves (ACC protoplasts), survival began to decline only after freezing to −6°C, with an LT_{50} of −9.3°C. In contrast, survival of *sfr4* ACC protoplasts began to decline at −3°C, with an LT_{50} of −5.6°C. Thus, a difference in freezing tolerance of *sfr4* mutant and wild-type protoplasts was evident only after cold acclimation.

The occurrence of the freeze-induced lesions, EIL and LOR, was analyzed after the freezing of protoplasts. Among protoplasts isolated from nonacclimated (NA) leaves (NA protoplasts), the incidence of EIL and LOR differed only slightly between *sfr4* and wild type (Fig. 2). EIL, despite its low incidence of

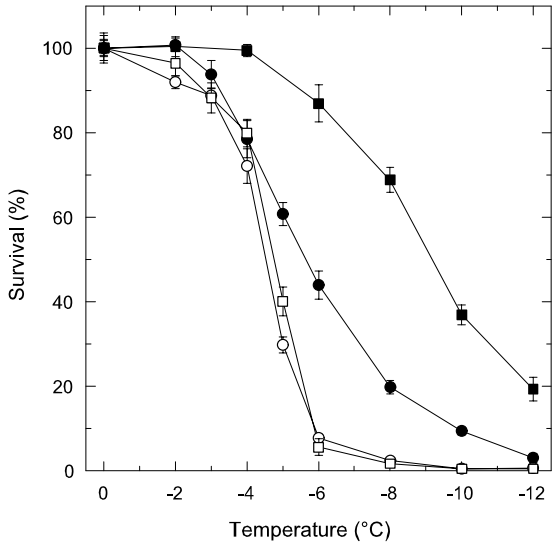

Figure 1. Effect of conventional cold acclimation on the survival of protoplasts isolated from leaves of seedlings of *sfr4* mutant and wild type. ○, Nonacclimated (NA) *sfr4* protoplasts; ●, 7-d-cold-acclimated (ACC) *sfr4* protoplasts; □, NA wild-type protoplasts; ■, 7-d-ACC wild-type protoplasts. Results (percent survival) are plotted relative to the survival of unfrozen controls. Each result indicates the mean and SD of at least two different experiments, with two samples taken per experiment and three counts performed on each sample. Absence of SD values indicates that they are smaller than the size of the symbols.

approximately 10%, was the predominant form of injury in both genotypes over the range of −2°C to −4°C, and was not observed below −5°C. The incidence of LOR, which was the predominant injury at the LT_{50} and below, increased from <5% to >90% over the range of −3°C to −6°C in both *sfr4* and the wild-type NA protoplasts.

Cold acclimation appeared to preclude EIL in both wild type and the *sfr4* mutant, leaving LOR as the predominant lesion at any injurious temperature (Fig. 2). However, the incidence was lower in wild type than in *sfr4* at any given temperature. The greatest differences in the incidence of LOR were observed at −6°C (12% in wild type versus 56% in *sfr4*) and −8°C (30% in wild type versus 79% in *sfr4*). These results indicated that although *sfr4* is able to ameliorate the incidence of EIL during cold acclimation, its ability to prevent LOR is substantially impaired, correlating with the large difference in freezing tolerance of the isolated protoplasts (see Fig. 1).

Effect of Suc Treatment on Freezing Tolerance

To examine the relationship between sugar deficiency and freezing sensitivity in the *sfr4* mutant after cold acclimation, experiments were carried out to determine whether Suc supplementation could restore freezing tolerance. Seedlings were incubated in a Suc solution (400 mM) at cold-acclimating temperature (2°C). The incubation was carried out in darkness to prevent the supply of carbohydrates from photosynthesis, which is presumably responsible for the differential accumulation of sugars in wild type versus *sfr4* under normal acclimating conditions. When *sfr4* seedlings were supplemented with Suc in this manner, a highly significant increase was observed in the freezing tolerance of the derived protoplasts (Fig. 3); this gain in freezing tolerance was much greater than what *sfr4* displayed after conventional cold acclimation. The survival of these *sfr4* protoplasts did not begin to decline until freezing to −5°C, 2°C lower than after the conventional cold acclimation. In contrast, in the wild type, Suc treatment increased freezing tolerance, but the increment was similar to that observed after the conventional cold acclimation. This implied that in wild type, intracellular sugar content is not a limiting factor for attaining maximum freezing tolerance. As a consequence, there was little difference in the freezing tolerance of protoplasts in wild type and *sfr4* after the Suc treatment (LT_{50} of −9.0°C). Thus, supplementation with Suc was fully successful in restoring the freezing tolerance of the *sfr4* mutant to wild-type levels.

This restoration implied that sugar deficiency was the primary cause of freezing sensitivity in the *sfr4* mutant. This interpretation would lead to the prediction that modes of freezing injury should become indistinguishable when the mutant was supplemented with Suc. In accordance, this prediction was tested by examining the types of freezing injury suffered by protoplasts derived from Suc-treated plants. In *sfr4* protoplasts, it was apparent that the incidence of both EIL and LOR was ameliorated (Fig. 2). Suc treatment completely precluded EIL over the range of −2°C to −4°C, which had also been observed in *sfr4* after conventional cold acclimation. However, the Suc treatment also reduced the incidence of LOR in *sfr4*, as had been observed in wild type but not *sfr4* after conventional cold acclimation. In wild type, the Suc treatment reduced the incidence of EIL and LOR, but the extents of their reductions were similar to those produced by conventional cold acclimation.

Freezing tolerance (estimated by survival) and the incidence of freeze-induced lesions were now followed over the time course of Suc treatment, assay-

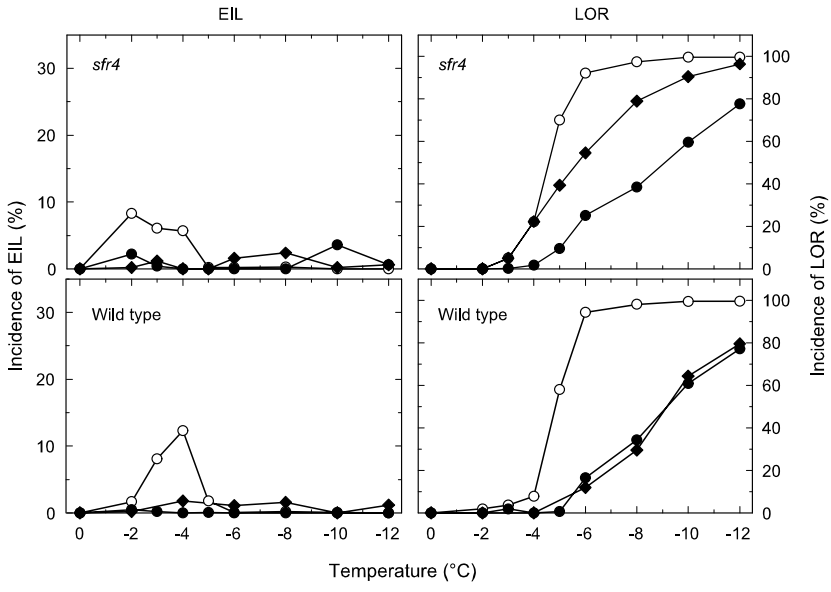

Figure 2. Effect of treatments on the incidence of EIL (left) and LOR (right) of protoplasts isolated from leaves of *sfr4* mutant and wild-type seedlings. ○, Protoplasts from NA plants (control); ●, protoplasts from plants subjected to conventional cold acclimation; ◆, protoplasts from plants that underwent Suc supplementation at low temperature in the dark (400 mM Suc, 2°C, 7 d). The difference in survival after the conventional freeze/thaw and the freeze/hypertonic thaw treatment is attributable to the incidence of EIL, and the difference in 100% survival and survival after the freeze/hypertonic thaw treatment is attributable to the incidence of LOR. SD of data was within ± 3.5%.

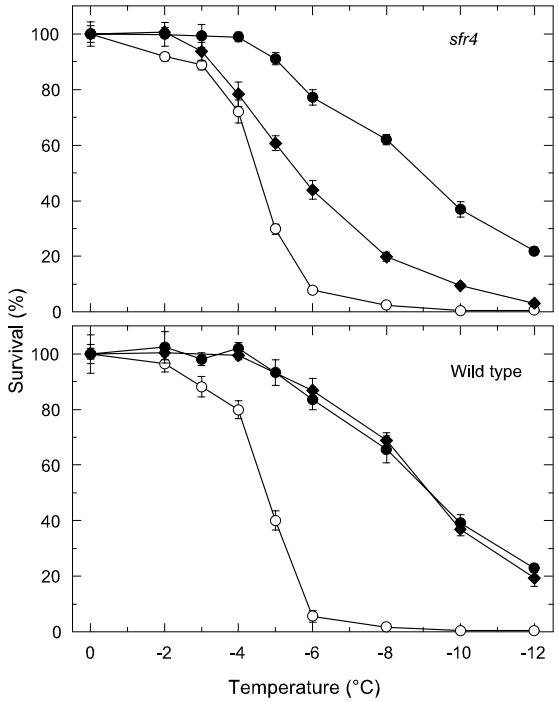

Figure 3. Effect of Suc treatment on survival of protoplasts isolated from leaves of *sfr4* mutant and wild-type seedlings. ○, Control (before Suc treatment); ●, after Suc treatment. For comparison, survival of protoplasts isolated after the conventional cold acclimation for 7 d (data were taken from Fig. 1) is included in the figure ◆.

ing at daily intervals. Both the increase in survival and the decrease in the incidences of EIL and LOR occurred more rapidly in wild type than in the *sfr4* mutant (Figs. 4 and 5). Freezing tolerance in wild type increased progressively but reached a plateau level at 5 d, equivalent to the level of tolerance produced by conventional cold acclimation. The incidence of EIL in the wild-type protoplasts was completely precluded at any injurious temperature by d 2 of the Suc treatment, and there was a progressive decrease in the incidence of LOR up to d 5 of the treatment. In the *sfr4* mutant, although there was a progressive increase through d 5 in the survival of temperatures between −5°C and −10°C, *sfr4* showed little change in survival of warmer temperatures (−2°C to −4°C) until d 4 of the Suc treatment. The incidence of EIL in the *sfr4* protoplasts did not begin to decrease until d 4 of the Suc treatment; it was precluded by d 5. The mutant's incidence of LOR at most freezing temperatures began to decline after 1 d of the Suc treatment. After 3 d, the incidence of LOR in *sfr4* was similar to that attained after 7 d of conventional cold acclimation, and by d 5, the incidence of LOR leveled off, showing no further decrease through d 7 of the Suc treatment.

DISCUSSION

In plants grown under nonacclimating conditions, it appears that there is little, if any, difference in the freezing characteristics of wild type and the *sfr4* mutant. The analysis of protoplasts in this study is consistent with the results of electrolyte leakage assays performed on intact leaves (Warren et al., 1996) in showing that *sfr4* and wild type have similar levels of freezing tolerance before cold acclimation. As expected, there was no difference in the incidence of EIL and LOR in protoplasts isolated from *sfr4* and wild type. Furthermore, McKown et al. (1996) found little or no difference between the genotypes in their boiling-soluble proteins, sugar content, or the fatty acid composition of leaf lipids in the absence of cold acclimation. Thus, the *sfr4* mutation appears to have little effect on the phenotypes associated with freezing tolerance in Arabidopsis plants grown under nonacclimating conditions.

On the other hand, there were substantial differences between *sfr4* and the wild type after cold acclimation. The freezing tolerance of isolated protoplasts increased in both, but the increase was much

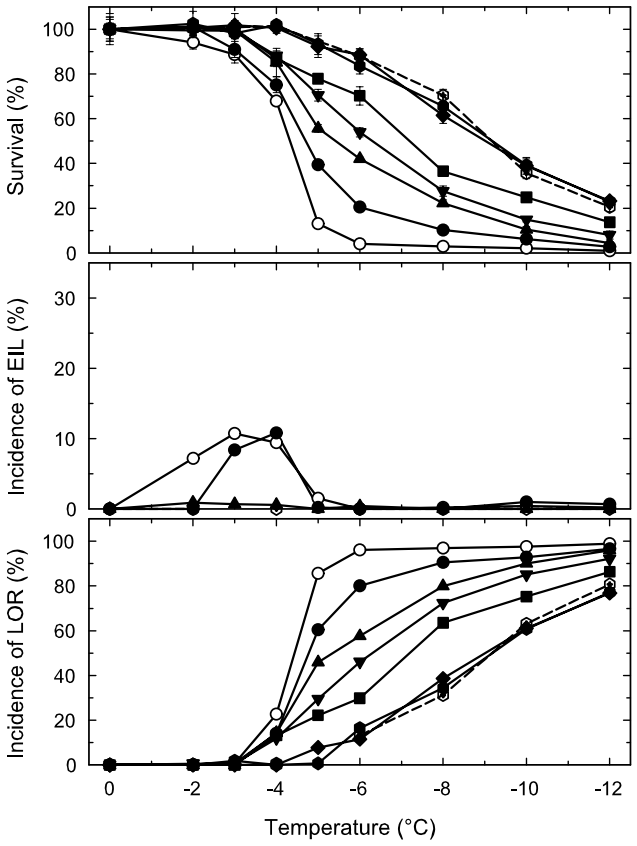

Figure 4. Determination of the effect of the Suc treatment on freezing tolerance of protoplasts isolated from leaves of wild-type seedlings at daily intervals. Survival (upper), the incidence of EIL (middle), and the incidence of LOR (lower) are shown. ○, Control (before Suc treatment); ●, Suc treatment for 1 d; ▲, Suc treatment for 2 d; ▼, Suc treatment for 3 d; ■, Suc treatment for 4 d; ◆, Suc treatment for 5 d; black hexagon, Suc treatment for 7 d. For comparison, freezing tolerance of protoplasts isolated after the conventional cold acclimation for 7 d (white hexagon with hair, taken from Figs. 1 and 2) is included in the figure.

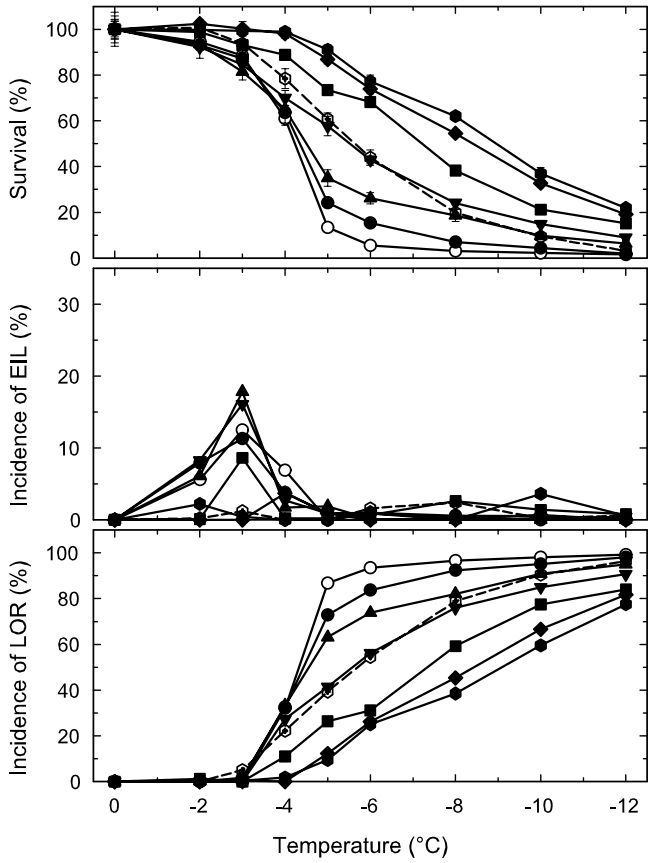

Figure 5. Determination of the effect of the Suc treatment on freezing tolerance of protoplasts isolated from leaves of *sfr4* mutant seedlings at daily intervals. Survival (upper), the incidence of EIL (middle), and the incidence of LOR (lower) are shown. For comparison, freezing tolerance of protoplasts isolated after the conventional cold acclimation for 7 d (data were taken from Figs. 1 and 2) is included in the figure. Symbols are the same as those in Figure 4.

less in *sfr4* mutant than in wild type (Fig. 1), as seen in intact leaves (Warren et al., 1996). Injury by EIL was completely eliminated by cold acclimation in wild-type and *sfr4* protoplasts. In contrast, the incidence of LOR, the predominant form of freezing injury in protoplasts isolated from Arabidopsis leaves after cold acclimation (Uemura et al., 1995), decreased in both (Fig. 2). However, the threshold temperature at which LOR was detected was significantly higher in *sfr4* protoplasts (−3°C) than in wild-type protoplasts (−6°C). Furthermore, at any given temperature the incidence of LOR was much greater in *sfr4* than wild type over the range of −4°C to −12°C. Therefore, the impairment of freezing tolerance in *sfr4* after cold acclimation appears to be attributable to a high incidence of LOR.

Because *sfr4* plants are deficient in the Glc and Suc contents after cold acclimation (McKown et al., 1996), we hypothesized that the reduced sugar content was responsible for the increase in LOR. We tested this by supplementation of *sfr4* plants with Suc at 2°C in the dark. The results presented in this study clearly showed that the Suc treatment decreased the incidence of LOR and, hence, restored the freezing tolerance of the *sfr4* mutant to a level shown by wild type after conventional cold acclimation. It was reported that incubation of Arabidopsis (ecotype RLD) seedlings in a 400 mM Suc at 2°C in the dark elevates in vivo sugars (Suc, Glc, and Frc) to levels well above those seen during conventional cold acclimation (Uemura and Steponkus, 1997, 2003), and a similar effect has been reproduced in a preliminary experiment on ecotype Columbia (data not shown). In addition, it was demonstrated that incubation of ecotype RLD seedlings with a Glc or Frc solution at 2°C in the dark also decreased the incidence of LOR, though the extent was less than that after incubation with a Suc solution (Uemura and Steponkus, 1997; 2003).

There might be alternative mechanisms that high levels of soluble sugars in the Suc-treated plants result in additional effects on the incidence of LOR. It has been reported that cold-regulated proteins (specifically COR15am) and membrane lipid composition as well as cytosolic sugars influence the incidence of LOR (Steponkus et al., 1993, 1998). It is possible that elevated sugar levels in the *sfr4* mutant might result in changes in *COR* gene expression and/or membrane lipid composition, which may in turn reduce the incidence of LOR. However, the *sfr4* mutant and wild type showed a normal inducibility of the *COR* genes by cold treatment (Knight et al., 1999). In addition, we considered that changes in membrane lipid composition induced during cold acclimation likely occur both in wild type and *sfr4* mutant (see below). It might be also possible that high levels of soluble sugars after the Suc treatment might mask the involvement of additional components that are deficient in the *sfr4* mutant and have an effect on the incidence of LOR. However, such components are still to be determined in future. Furthermore, the wild type and *sfr4* mutant have markedly similar LOR spectra after Suc treatment (Figs. 4 and 5). Therefore, we believe at this moment that the results in the present study support the notion that reduced sugar content apparently is associated with a high incidence of LOR in ACC *sfr4* plants and, thus, is responsible for the freezing sensitivity of the mutant.

After cold acclimation, besides showing a reduction in its content of soluble sugars, the *sfr4* mutant has been observed to differ from wild type in the fatty acid composition of total leaf lipids (McKown et al., 1996). Such lipids contained lower proportions of palmitic acid (16:0), oleic acid (18:1), and linoleic acid (18:2) than wild type. The possibility must be considered that this difference (rather than or in addition to reduced sugar content) might be responsible for the freezing tolerance deficiency of the mutant. The following considerations argue against this. First, the occurrence of EIL, which is known to be sensitive to the lipid composition of the plasma membrane, was still completely precluded in the *sfr4* mutant after

cold acclimation (Fig. 2). Cold acclimation produces a substantial increase in the proportion of phospholipids in the plasma membrane in various plant species, including rye (*Secale cereale*; Uemura and Yoshida, 1984; Lynch and Steponkus, 1987), orchard grass (*Dactylis glomerata*; Yoshida and Uemura, 1984), oat (*Avena sativa*; Uemura and Steponkus, 1994), and Jerusalem artichoke (*Helianthus tuberosus*; Ishikawa and Yoshida, 1985). Membrane engineering studies (involving manipulation of the plasma membrane lipid composition by protoplast-liposome fusion) revealed that an increase in the proportion of unsaturated species of phosphatidylcholine causes a decrease in the incidence of EIL (Steponkus et al., 1988; Uemura and Steponkus, 1989). Thus, alterations in the plasma membrane lipid composition induced by low temperatures appear both necessary and sufficient to reduce the incidence of EIL. Because EIL in the *sfr4* mutant was precluded by cold acclimation, it seems likely that alterations in the lipid composition of the plasma membrane in the *sfr4* mutant are equivalent to those in wild type. Second, the earlier study on fatty acid composition of *sfr4* did not entail subcellular fractionation or discrimination between molecular species of lipids. Therefore, there is no evidence that *sfr4* has any effect on the lipid content of the particular membranes (plasma membrane and chloroplast envelope) whose interaction results in a series of ultrastructural changes in these two membranes, including the hexagonal II phase formation and, hence, leads to the occurrence of LOR. The lipid component of the chloroplast envelope responsible for LOR, monogalactosyldiacylglycerol, is known to be highly unsaturated even before cold acclimation (Douce et al., 1984). Overall, these arguments do not support the possibility that the difference in fatty acid composition in *sfr4* mutant and wild type is responsible for its susceptibility to LOR after cold acclimation. Because the putative low-temperature-induced alterations of lipid composition associated with the decreased propensity of EIL and LOR are likely to occur in the *sfr4* mutant under a conventional cold acclimation as described above, it is unlikely that the incubation of *sfr4* seedlings in a Suc solution at 2°C in the dark results in additional changes in lipid composition that is necessary for the decrease in LOR. Nevertheless, it would be desirable to elucidate whether Suc supplementation eliminates the difference in total lipid composition in ACC *sfr4* plants.

In many plants, including Arabidopsis, the occurrence of LOR is associated either with freeze-induced formation of the hexagonal II phase before cold acclimation or with the fracture-jump lesion after cold acclimation (Steponkus et al., 1993; Uemura et al., 1995). The occurrence of the fracture-jump lesion appears to be determined by the closeness of apposition of the plasma membrane with other endomembranes (often the chloroplast envelope) during freeze-induced dehydration (Steponkus et al., 1993). Increased content of compatible solutes such as soluble sugars would, at any given freezing temperature, reduce the apposition of membranes; thus, it is entirely plausible that LOR would result from low sugar levels in the *sfr4* mutant and be prevented when sugar levels were artificially raised.

Differential scanning calorimetric studies of the effect of sugars on the lyotropic phase behavior and lamellar-to-hexagonal II phase transition of 1,2-dioleoylphosphatidylethanolamine, a non-bilayer-forming lipid in the plasma membrane, revealed that Frc and Suc decrease the propensity of the dehydration-induced hexagonal II phase formation at moderate dehydration level (Shalaev and Steponkus, 2001). When 1,2-dioleoylphosphatidylethanolamine, a major species of phosphatidylethanolamine in the plasma membrane, is dehydrated in the presence of Frc or Suc at pressures up to 67 MPa, there is no detectable lamellar-to-hexagonal II phase transition during heating to 35°C. Rather, 1,2-dioleoylphosphatidylethanolamine remains in the lamellar phase. Although it is necessary to determine the effect of sugars on the occurrence of the fracture-jump lesion, which is the primary freeze-induced lesion occurring in ACC protoplasts of Arabidopsis plants, these results strongly suggest that sugars tend to oppose the dehydration-induced destabilization of membranes. Therefore, it is physicochemically plausible that the lack of sugar accumulation in the cytoplasm of *sfr4* cells during cold acclimation is largely responsible for a high incidence of LOR, and, hence, the impaired increase in freezing tolerance.

Finally, these studies also suggest that the *sfr4* mutant might be useful as a tool to study the molecular mechanism of cold acclimation in plants. Because the mutant's freezing sensitivity is primarily due to sugar deficiency and resulted from higher incidence of a specific freeze-induced lesion (i.e. LOR), it may offer a more sensitive test system for the protective effects of other osmolytes and for the particular impact of various transgenes on injury caused by LOR.

MATERIALS AND METHODS

Plant Materials and Cold Acclimation

We utilized Columbia (wild type) and FR67 (a homozygous *sfr4* segregant from the cross of FS67 × Columbia; its background is pure Columbia) lines of Arabidopsis. Seeds were planted in a clay pot (130 mm in diameter) filled with moist Metro Mix 350 (Scotts-Sierra, Maysville, OH). Seedlings were grown in a controlled environment chamber at 23°C under continuous illumination (light intensity = 150 μmol m^{-2} s^{-1} at soil level) and irrigated as necessary from the bottom with Hoagland solution. NA plants remained in this environment for 17 to 20 d. Cold acclimation was achieved by transferring 17- to 20-d-old plants to a 2°C-environment (8-h photoperiod, 125 μmol m^{-2} s^{-1}) for 7 d. For protoplast isolation, the third to fifth leaves of seedlings were excised and immediately used.

Suc Treatment of Seedlings

Before incubation in Suc, NA seedlings in clay pots (17 to 20 d old) were held in darkness for 24 h at 23°C to deplete the endogenous sugar and starch

levels (Steponkus and Lanphear, 1968). After the dark treatment, seedlings were carefully removed from soil, washed three times with distilled water, and wiped with a Kimwipe (Kimberly-Clark, Irving, TX) to remove excess water; these manipulations were performed under minimal light. The seedlings were then placed in a plastic petri dish (150 mm in diameter) containing a 400 mM Suc solution. The petri dish was covered with a lid, wrapped with aluminum foil, and then incubated at 2°C for 7 d. After this treatment, leaves were excised and used for protoplast isolation immediately.

Protoplast Isolation

Protoplasts were enzymatically isolated from leaves according to the method described previously (Uemura et al., 1995) except that a sorbitol solution with a higher osmolality (0.6 osmol) was employed. This was because the solute concentration of cells in leaves after the Suc treatment was expected to be increased and, as a result, isolated protoplasts had to be maintained in a solution in a higher osmolality to avoid exposing the protoplasts to hypotonic conditions. The plasmolysis test of the leaf segments after Suc treatment revealed that 0.6 osmol was the appropriate osmolality (slightly hypertonic) to isolate and suspend protoplasts from leaves of seedlings (compare with 0.4 osmol for NA leaves; Uemura et al., 1995), which is similar to that of 7-d-acclimated leaves of Columbia. Survival of protoplasts of wild type isolated and frozen in a 0.6 osmol sorbitol solution (Fig. 1) was similar to that of protoplasts isolated and frozen in a 0.4 osmol sorbitol solution (Uemura et al., 1995). From these results, we chose to isolate protoplasts from all samples employed under the same conditions, i.e. in a 0.6 osmol sorbitol solution.

For protoplast isolation, 15 to 20 excised leaves were washed with distilled water three times and then thoroughly blotted dry with a Kimwipe paper. Leaves were trisected and placed in 12.5 mL of enzyme solution, consisting of 1.2% (w/v) cellulysin (Calbiochem-Novabiochem, San Diego), 0.4% (w/v) macerase (Calbiochem-Novabiochem), 0.6 osmol sorbitol, 1 mM $CaCl_2$, and 10 mM MES/KOH (at pH 5.5). After in vacuo treatment for several minutes, incubation in the enzyme solution was continued for 2 h at 27°C in the dark with a gentle shaking. After incubation, undigested leaf sections were removed by filtration through four layers of cheesecloth. The filtrate was centrifuged at 50*g* for 10 min at 4°C to collect the protoplasts. The pellet was suspended in a 0.6 osmol sorbitol solution containing 1 mM $CaCl_2$ and 1 mM MES/KOH (pH 5.5) and then washed twice by centrifugation and resuspending. The washed protoplasts were suspended in the sorbitol solution containing 1 mM $CaCl_2$ and 1 mM MES/KOH (pH 5.5) and kept on ice. The isolated protoplasts were immediately used in experiments.

Determination of Protoplast Freezing Tolerance

Freezing of protoplasts was performed as described previously (Uemura et al., 1995). In brief, an aliquot of the protoplast suspension (0.2 mL, 4×10^5 protoplasts) in a test tube (10 × 100 mm) was placed in an ethanol bath (ULT-80, Neslab, Portmouth, NH) at −2°C for 15 min before ice nucleation. Ice nucleation was performed by touching a spatula precooled in liquid nitrogen to the wall of the test tube. After an additional 1-h incubation at −2°C, the samples were cooled to the specified temperatures at a rate of 0.8°C min^{-1}. After 2.5 h at the specified temperatures, the samples were thawed at room temperature and then kept on ice immediately. This freeze/thaw protocol was referred to as a conventional freeze/thaw treatment. To determine the incidence of EIL and LOR, a hypertonic/thaw treatment was employed to minimize osmotic expansion of protoplasts during thawing of the suspension medium (Steponkus et al., 1988; Uemura and Steponkus, 1989). For this, the frozen suspensions (except those frozen to −2°C) were warmed to −3°C for 5 min, after which a hypertonic sorbitol solution (0.25 mL) containing 1 mM $CaCl_2$ and 10 mM MES/KOH (pH 5.5) at −3°C was added to the suspensions. This procedure yielded a final osmolality of 1.61 after melting of ice in the suspension. The suspensions frozen to −2°C were kept at −2°C during thawing and addition of the hypertonic sorbitol solution to yield the final osmolality of 1.08. After ice had melted, protoplast suspensions were kept on ice.

Survival of protoplasts was determined by staining with fluorescein diacetate (Widholm, 1972). After the samples were incubated with fluorescein diacetate for 5 to 10 min at room temperature, the number of protoplasts that retained fluorescein was counted in a hemocytometer. Typically, in the unfrozen control, 150 to 200 protoplasts were counted in each of the hemocytometer fields; three hemocytometer samples were counted for each treatment of a given temperature (450–600 protoplasts). Survival of frozen samples was expressed as a percentage of the unfrozen control. The results shown were the average and SD, if shown, of, at least, two experiments.

The incidences of EIL and LOR were calculated with the survival percentages after a conventional freeze/thaw treatment and a freeze/hypertonic thaw treatment (Steponkus et al., 1988; Uemura and Steponkus, 1989). The difference in survival between the conventional freeze/thaw treatment and the freeze/hypertonic thaw treatment is attributable to the incidence of EIL. The incidence of LOR is obtained as the difference between 100% survival and survival after the freeze/hypertonic thaw treatment.

Received August 16, 2002; returned for revision September 7, 2002; accepted December 31, 2002.

LITERATURE CITED

Douce R, Block MA, Dorne A-J, Joyard J (1984) The plastid envelope membranes: their structure, composition, and role in chloroplast biogenesis. *In* DB Roodyn ed, Subcellular Biochemistry, Vol 10. Plenum Press, New York, pp 1–84

Gilmour SJ, Thomashow MF (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol Biol **17:** 1233–1240

Green DC, Ratzlaff CD (1975) An apparent relationship of soluble sugars with hardiness in winter wheat varieties. Can J Bot **53:** 2198–2201

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223

Heino P, Sandman G, Lang V, Nordin K, Palva ET (1990) Abscisic acid deficiency prevents development of freezing tolerance in *Arabidopsis thaliana* L. Heynh. Theor Appl Genet **79:** 801–806

Ishikawa M, Yoshida S (1985) Seasonal changes in plasma membranes and mitochondria isolated from Jerusalem artichoke tubers: possible relationships to cold hardiness. Plant Cell Physiol **26:** 1331–1344

Knight H, Veale EL, Warren GJ, Knight MR (1999) The *sfr6* mutation in *Arabidopsis* suppresses low-temperature induction of genes dependent in the CRT/DRE sequence motif. Plant Cell **11:** 875–886

Koster KL, Lynch DV (1992) Solute accumulation and compartmentation during the cold acclimation of puma rye. Plant Physiol **98:** 108–113

Lee H, Xiong L, Ishitani M, Stevenson B, Zhu JK (1999) Cold-regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant. Plant J **17:** 301–308

Levitt J (1980) Responses of Plants to Environmental Stresses, Ed 2. Academic Press, New York

Lynch DV, Steponkus PL (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **83:** 761–767

Mäntylä E, Lång V, Palva ET (1995) Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LTI78 and RAB18 proteins in *Arabidopsis thaliana*. Plant Physiol **107:** 141–148

McKown R, Kuroki G, Warren G (1996) Cold responses of *Arabidopsis* mutants impaired in freezing tolerance. J Exp Bot **47:** 1919–1925

Ristic Z, Ashworth EN (1993) Changes in leaf ultrastructure and carbohydrates in *Arabidopsis thaliana* L. (Heynh) cv. Columbia during rapid cold acclimation. Protoplasma **172:** 111–123

Sakai A (1962) Studies on the frost-hardiness of woody plants: I. The causal relation between sugar content and frost-hardiness. Cont Inst Low Temp Sci B **11:** 1–40

Sakai A, Larcher W (1987) Frost Survival of Plants: Responses and Adaptation to Freezing Stress. Springer-Verlag, Berlin

Sasaki H, Ichimura K, Oda M (1996) Changes in sugar content during cold acclimation and deacclimation of cabbage seedlings. Ann Bot **78:** 365–369

Shalaev EY, Steponkus PL (2001) Phase behavior and glass transition of 1,2-dioleoylphosphatidylethanolamine (DOPE) dehydrated in the presence of sucrose. Biochim Biophys Acta Biomembr **1514**: 100–116

Siminovitch D (1981) Common and disparate elements in the processes of adaptation of herbaceous and woody plants to freezing: a perspective. Cryobiology **18:** 166–185

Steponkus PL (1984) Role of plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Steponkus PL, Lanphear FO (1968) The relationship of carbohydrates to cold acclimation of *Hedera helix L. cv. Thorndale*. Physiol Plant **21:** 777–791

Steponkus PL, Uemura M, Balsamo RA, Arvinte T, Lynch DV (1988) Transformation of the cryobehavior of rye protoplasts by modification of the plasma membrane lipid composition. Proc Natl Acad Sci USA **85:** 9026–9030

Steponkus PL, Uemura M, Webb MS (1993) A contrast of the cryostability of the plasma membrane of winter rye and spring oat-two species that widely differ in their freezing tolerance and plasma membrane lipid composition. *In* PL Steponkus, ed, Advances in Low-Temperature Biology, Vol 2. JAI Press, Ltd., London, pp 211–312

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **95:** 14570–14575

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol **50:** 571–599

Tumanov II, Trunova TI (1963) First phase of frost hardening of winter wheat in the dark on sugar solutions. Sov Plant Physiol **10:** 140–149

Uemura M, Joseph RA, Steponkus PL (1995) Cold acclimation of *Arabidopsis thaliana*: effect on plasma membrane lipid composition and freeze-induced lesions. Plant Physiol **109:** 15–30

Uemura M, Steponkus PL (1989) Effect of cold acclimation on the incidence of two forms of freezing injury in protoplasts isolated from rye leaves. Plant Physiol **91:** 1131–1137

Uemura M, Steponkus PL (1994) A contrast of the plasma membrane lipid composition of spring oat and winter rye leaves in relation to freezing tolerance. Plant Physiol **104:** 479–496

Uemura M, Steponkus PL (1997) Artificial manipulation of the intracellular sucrose content alters the incidence of freeze-induced membrane lesions of isolated protoplasts of *Arabidopsis thaliana*. Cryobiology **35:** 336

Uemura M, Steponkus PL (2003) Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. Plant Cell Environ (in press)

Uemura M, Yoshida S (1984) Involvement of plasma membrane alterations in cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **75:** 818–826

Wanner L, Junttila O (1999) Cold-induced freezing tolerance in *Arabidopsis*. Plant Physiol **120:** 391–399

Warren G, McKown R, Marin A, Teutonico R (1996) Isolation of mutants affecting the development of freezing tolerance in *Arabidopsis thaliana* (L.) Heynh. Plant Physiol **111:** 1011–1019

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol **47:** 189–194

Yoshida M, Abe J, Moriyama M, Kuwabara T (1998) Carbohydrate levels among winter wheat cultivars varying in freezing tolerance and snow mold resistance during autumn and winter. Plant Physiol **103:** 8–16

Yoshida S, Uemura M (1984) Protein and lipid compositions of isolated plasma membranes from orchard grass (*Dactylis glomerata* L.) and changes during cold acclimation. Plant Physiol **75:** 31–37